Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for the Titration of Feline Panleukopenia Virus in Cell Culture

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1. Introduction

1.1 Background

This Supplemental Assay Method (SAM) describes an *in vitro* test method for assaying modified-live feline panleukopenia virus (FPV) vaccines for viral content. The method uses the Crandall feline kidney (CRFK) cell line as the test system. Presence or absence of FPV is determined by staining inoculated cell cultures by an indirect fluorescent antibody (IFA) technique.

1.2 Keywords

FPV, IFA, *in vitro*, cell culture, viral titration

2. Materials

2.1 Equipment/instrumentation

2.1.1 Incubator,[1] $36^0 \pm 2^0$C, high humidity, 5 ± 1% CO_2 (CO_2 incubator) meeting the requirements of the current version of GDOCSOP0004

2.1.2 Incubator,[2] aerobic

2.1.3 Water bath[3] meeting the requirements of the current version of GDOCSOP0002

2.1.4 Ultraviolet (UV) light microscope[4]

2.1.5 Vortex mixer[5]

2.1.6 Micropipettor[6] and/or motorized microliter pipette[7] and tips[8]

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[1] Model 3336, Forma Scientific, Inc., P.O. Box 649, Marietta, OH 45750 or equivalent

[2] Model 2, Precision Scientific, 3737 West Cortland St., Chicago, IL 60647 or equivalent

[3] Cat. No. 66648, Precision Scientific or equivalent

[4] Model BH2, Olympus America, Inc., 2 Corporate Ctr. Dr., Melville, NY 11747 or equivalent

[5] Vortex-2 Genie, Model G-560, Scientific Industries, Inc., 70 Orville Dr., Bohemia, NY 11716 or equivalent

[6] Cat. No. P-200, Rainin Instrument Co., P.O. Box 4026, Mack Rd., Woburn, MA 01801-4628 or equivalent

[7] Cat. No. E2-1000, Rainin Instrument Co. or equivalent

[8] Cat. No. YE-3R, Analytic Lab Accessories, P.O. Box 345, Rockville Center, NY 11571 or equivalent

2.1.7 Microscope slide, glass staining dish with rack (glass staining dish)[9]

2.2 Reagents/supplies

2.2.1 FPV Reference,[10] ICK strain

2.2.2 CRFK cell culture,[11] free of extraneous agents as tested by the Code of Federal Regulations, Title 9 (9 CFR)

2.2.3 FPV Antiserum[12]

2.2.4 Minimum essential medium (MEM)

2.2.4.1 9.61 g MEM with Earle's salts without bicarbonate[13]

2.2.4.2 2.2 g sodium bicarbonate ($NaHCO_3$)[14]

2.2.4.3 Q.S. to 1000 ml with deionized water (DW); adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).[15]

2.2.4.4 Sterilize through a 0.22-µm filter.[16]

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[9] Cat. No. 121, Shandon Lipshaw, 171 Industry Dr., Pittsburg, PA 15275 or equivalent

[10] Seed quantities available upon request from the Center for Veterinary Biologics-Laboratory (CVB-L), P.O. Box 844, Ames, IA 50010 or equivalent

[11] CCL-94, American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD 20852-1776

[12] Reference quantities available upon request from the CVB-L or equivalent

[13] Cat. No. 410-1500EF, Life Technologies, Inc., 8400 Helgerman Ct., Gaithersburg, MD 20884 or equivalent

[14] Cat. No. S-5761, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178 or equivalent

[15] Cat. No. 9535-01, J.T. Baker, Inc., 222 Red School Ln., Phillipsburg, NJ 08865 or equivalent

[16] Cat. No. 12122, Gelman Sciences, 600 S. Wagner Rd., Ann Arbor, MI 48106 or equivalent

2.2.4.5 Aseptically add:

1. 25 units/ml penicillin[17]

2. 50 µg/ml gentamicin sulfate[18]

3. 100 µg/ml streptomycin[19]

2.2.4.6 Store at $4^0 \pm 2^0$C.

2.2.5 Growth Medium

2.2.5.1 920 ml MEM

2.2.5.2 Aseptically add:

1. 70 ml gamma irradiated fetal bovine serum

2. 10 ml L-glutamine[20]

2.2.6 Dulbecco's phosphate buffered saline (DPBS)

2.2.6.1 8.0 g sodium chloride (NaCl)[21]

2.2.6.2 0.2 g potassium chloride (KCl)[22]

2.2.6.3 0.2 g potassium phosphate, monobasic, anhydrous (KH_2PO_4)[23]

2.2.6.4 0.1 g magnesium chloride, hexahydrate ($MgCl_2 \cdot 6H_2O$)[24]

2.2.6.5 Dissolve reagents in 900 ml DW.

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[17]Cat. No. 0049-0530-28, Schering Laboratories, 2000-T Galloping Hill Rd., Kenilworth, NJ 07033 or equivalent

[18]Gentocin® solution, Cat. No. 0061-0464-04, Schering Laboratories or equivalent

[19]Cat. No. S-9137, Sigma Chemical Co. or equivalent

[20]L-glutamine-200 mM (100X), liquid, Cat. No. 320-503PE, Life Technologies, Inc. or equivalent

[21]Cat. No. 3624-01, J.T. Baker, Inc. or equivalent

[22]Cat. No. P217-500, Fisher Scientific Corp., 2000 Park Ln., Pittsburgh, PA 15275 or equivalent

[23]Cat. No. 3246-01, J.T. Baker, Inc. or equivalent

[24]Cat. No. M33-500, Fisher Scientific Corp. or equivalent

2.2.6.6 Dissolve 1.03 g sodium phosphate, dibasic, anhydrous (Na_2HPO_4)[25] with 10 ml DW, heat to $60^0 \pm 2^0$C until dissolved, then add to **Section 2.2.6.5** with constant mixing.

2.2.6.7 Dissolve 0.1 g calcium chloride, anhydrous ($CaCl_2$)[26] with 10 ml DW and add slowly to **Section 2.2.6.6** to avoid precipitation.

2.2.6.8 Q.S. to 1000 ml with DW, adjust pH to 7.0-7.3 with 2N HCl.

2.2.6.9 Sterilize through a 0.22-µm filter.

2.2.7 Glass slides, 8 chamber[27] (Lab-Tek® slides)

2.2.8 Polystyrene tubes, 12 x 75 mm[28]

2.2.9 Goat anti-cat IgG (H&L) fluorescein isothiocyanate labeled conjugate (Goat Anti-cat Conjugate)[29]

2.2.10 100% Acetone[30]

2.2.11 Syringe, 3 ml[31] and needle, 20 ga x 1.5 in[32]

2.2.12 Self-refilling repetitive syringe, 2 ml[33]

2.2.13 Pipette-aid[34]

2.2.14 Disposable transfer pipette, 3.5 ml[35]

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[25]Cat. No. 3828-01, J.T. Baker, Inc. or equivalent

[26]Cat. No. 4225-05, J.T. Baker, Inc. or equivalent

[27]Cat. No. 177402, Nunc, Inc., 2000 N. Aurora Rd., Naperville, IL 60563 or equivalent

[28]Falcon 2058, Becton Dickinson Labware, 1 Becton Dr., Franklin Lakes, NJ 07417 or equivalent

[29]Cat. No. 102-015-003, Jackson ImmunoResearch Laboratories, Inc., 872 W. Baltimore Pike, West Grove, PA 19390 or equivalent

[30]Cat. No. A18-4, Fisher Scientific Corp. or equivalent

[31]Luer-Lok®, Cat. No. 309585, Becton Dickinson Labware or equivalent

[32]Cat. No. 250107, Becton Dickinson Labware or equivalent

[33]Wheaton, Cat. No. 13-689-50C, Fisher Scientific Co., 319 W. Ontario, Chicago, IL 60610 or equivalent

[34]Cat. No. 183, Drummond Scientific Co., 500 Pkwy., Broomall, PA 19008 or equivalent

[35]Cat. No. P600C, Columbia Diagnostics, Inc., 8001 Research Way, Springfield, VA 22153 or equivalent

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3. Preparation for the test

3.1 Personnel qualifications/training

Personnel shall have experience in the preparation and maintenance of cell culture as well as in the propagation of animal viruses and the quantitation of virus infectivity by IFA.

3.2 Preparation of equipment/instrumentation

3.2.1 On the day of initial titration, set a water bath at $56^0 \pm 2^0$C.

3.2.2 On the day of initial titration, set a water bath at $36^0 \pm 2^0$C.

3.2.3 On the day of the IFA test, prepare a humidity chamber in the aerobic incubator by filling a pan in the bottom with DW.

3.3 Preparation of reagents/control procedures

3.3.1 Preparation of CRFK slides

3.3.1.1 Cells are prepared from healthy, confluent CRFK cells, that are maintained by splitting every 3 to 4 days. On the day of test initiation, using a 2-ml self-refilling repetitive syringe, add 0.4 ml/well of approximately $10^{5.2} \pm 10^{4.9}$ cells/ml diluted in Growth Medium into all wells of the Lab-Tek® Slides. Prepare sufficient Lab-Tek® Slides to allow 25 wells for controls and 20 wells for each Test Serial. Incubate at 37° ± 2°C in a CO_2 incubator and use within 4 hr. These become the CRFK Slides.

3.3.1.2 Use seeded CRFK Slides within 4 hr.

3.3.2 Preparation of Working FPV Reference

3.3.2.1 On the day of inoculation, rapidly thaw a vial of FPV Reference in a $36^0 \pm 2^0$C water bath.

3.3.2.2 Using the self-refilling repetitive syringe, dispense 1.8 ml of MEM into each of 7, 12 x 75-mm polystyrene tubes labeled 10^{-1} through 10^{-7}.

3.3.2.3 Transfer 200 µl of the FPV Reference to the tube labeled 10^{-1}; discard pipette tip. Mix by vortexing.

3.3.2.4 Transfer 200 µl from the 10^{-1} labeled tube to the 10^{-2} tube; discard pipette tip. Mix by vortexing.

3.3.2.5 Repeat **Section 3.3.2.4** for each of the subsequent dilutions, transferring 200 µl from the previous dilution to the next dilution.

3.3.3 Preparation of Working FPV Antiserum

On the day of the IFA test, dilute FPV Antiserum in DPBS to the IFA working dilution determined for that specific antiserum.

3.3.4 Preparation of Working Goat Anti-cat Conjugate

On the day of the IFA test, dilute Goat Anti-cat Conjugate in DPBS to the working dilution according to the manufacturer’s recommendations.

3.4 Preparation of the sample

3.4.1 The initial test of a Test Serial will be with a single vial (a single sample from 1 vial). On the day of inoculation, rehydrate a vial of the Test Serial by transferring 1.0 ml for 1-ml dose vaccine, 0.5 ml for 1/2-ml dose vaccine, etc. of the provided diluent into the vial containing the lyophilized Test Serial. Use a sterile 1.0-ml syringe and an 18-ga x 1.5-in needle;

mix by vortexing. Incubate for 15 ± 5 min at room temperature (RT) (23^0 ± 2^0C).

3.4.2 For a monovalent FPV Test Serial, mix 200 µl of Test Serial with 1.8 ml of MEM in a 12 x 75-mm polystyrene tube for a 10^{-1} dilution. Mix by vortexing.

3.4.3 For a multifraction Test Serial containing FPV, heat inactivate the non-FPV fraction(s) in the Test Serial in a 56^0 ± 2^0C water bath for 60 ± 5 min. Dilute according to **Section 3.4.2**.

3.4.4 Dispense 1.8 ml MEM into each of 5, 12 x 75-mm polystyrene tubes labeled 10^{-2} through 10^{-6}.

3.4.5 Transfer 200 µl from the tube labeled 10^{-1} to the tube labeled 10^{-2}; discard pipette tip. Mix by vortexing.

3.4.6 Repeat **Section 3.4.5** for each subsequent dilution, transferring 200 µl from the previous dilution to the next dilution.

4. Performance of the test

4.1 Inoculate 5 chambers/dilution of a CRFK Slide with 100 µl/chamber from dilutions 10^{-6} through 10^{-3} of the Test Serial. Tip changes are not necessary between each dilution in a series if pipetting from the most dilute to the most concentrated within that series (e.g. 10^{-6} through 10^{-3}).

4.2 Inoculate 5 chambers/dilution of a CRFK Slide with 100 µl/chamber, from dilutions 10^{-6} through 10^{-3} of the Working FPV Reference. Tip changes are not necessary between each dilution in a series if pipetting from the most dilute to the most concentrated within that series (e.g. 10^{-6} through 10^{-3}).

4.3 Five uninoculated chambers serve as a negative cell control.

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4.4 Incubate CRFK Slides in a $36^0 \pm 2^0$C CO_2 incubator for 120 ± 12 hr.

4.5 Following incubation, decant the media from the CRFK Slide and remove the plastic wall by twisting them away from the CFRK Slide, leaving the gasket attached to the CRFK Slide.

4.6 Place the CRFK Slides in a slide rack; place the rack in a glass staining dish filled with DPBS. Let stand 15 ± 5 min at RT.

4.7 Discard the DPBS and fix the CRFK Slides in 100% Acetone for 15 ± 5 min at RT. Remove and allow to air dry.

4.8 Pipette 75 ± 25 µl of the Working FPV Antiserum into each chamber of the CRFK Slides with a transfer pipette. Incubate in the aerobic incubator at $36^0 \pm 2^0$C for 30 ± 5 min.

4.9 Wash per **Section 4.6**. Discard the DPBS.

4.10 Pipette 75 ± 25 µl of the Working Goat Anti-cat Conjugate into each chamber of the CRFK Slides; incubate in the aerobic incubator at $36^0 \pm 2^0$C for 30 ± 5 min.

4.11 Wash per **Section 4.6**. Discard the DBPS.

4.12 Rinse the CRFK Slides with DW, allow to air dry.

4.13 Read at 100-200X with a UV-light microscope. Examine the cell monolayer for typical apple-green nuclear fluorescence.

4.13.1 Chambers containing 1 or more cells displaying specific fluorescence for FPV are positive.

4.13.2 Results are recorded as the number of IFA positive chambers versus total number of chambers examined for each dilution of a Test Serial and the Working FPV Reference.

4.14 Calculate the FPV endpoints of the Test Serial and the Working FPV Reference using the Spearman-Kärber method as modified by Finney. The titer is expressed as $\log_{10}$ 50% tissue culture infective dose ($TCID_{50}$).

Example:

10^{-3} dilution of Test Serial = 5/5 chambers IFA pos.
10^{-4} dilution of Test Serial = 5/5 chambers IFA pos.
10^{-5} dilution of Test Serial = 2/5 chambers IFA pos.
10^{-6} dilution of Test Serial = 0/5 chambers IFA pos.

Spearman-Kärber calculation of total IFA positive wells (12),

Using 5 wells per dilution	=1.9 log
$\log_{10}$ of reciprocal dilution (10^{-3})	=3.0 log
$\log_{10}$ of reciprocal of dose factor:	
$\frac{0.1 \text{ ml inoculum}}{1\text{-ml dose}} = \frac{1}{10}$	=1.0 log
Total	=5.9 log

Titer of the Test Serial is $10^{5.9}$ $TCID_{50}$ per 1-ml dose.

5. Interpretation of the test results

5.1 Validity requirements:

5.1.1 The calculated titer of the Working FPV Reference must fall within ± 2 standard deviations (SD) of its mean titer, as established from a minimum of 10 previously determined titers.

5.1.2 The uninoculated cell controls must not exhibit any cytopathic effect, specific FPV fluorescence, or cloudy media that would indicate contamination.

5.1.3 The lowest inoculated dilution must exhibit a 100% positive IFA reaction (5/5), and the highest (most dilute) must exhibit a negative IFA reaction (0/5).

5.1.4 If the validity requirements are not met, then the assay is considered a NO TEST and can be retested without prejudice.

5.1.5 If the validity requirements are met and the titer of the Test Serial is greater than or equal to the titer contained in the Animal and Plant Health Inspection Service (APHIS) filed Outline of Production for the product under test, the Test Serial is considered SATISFACTORY.

5.1.6 If the validity requirements are met but the titer of the Test Serial is less than the required minimum titer contained in the APHIS filed Outline of Production for the product under test, the Test Serial is retested in accordance with 9 CFR 113.8.

6. Report of test results

Results are reported as the $TCID_{50}$ per dose.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.304, U.S. Government Printing Office, 1999.

7.2 Cottral G.E., (Ed.), 1978. *Manual of standardized methods for veterinary microbiology*. Comstock Publishing Associates, Ithaca, NY. pg. 731.

7.3 Finney, D.J. 1978. *Statistical methods in biological assay.* Griffin, London. 3rd edition, pg. 508.

8. Summary of revisions

This document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use in the CVB-L, and to provide additional detail. No significant changes were made from the previous protocol.